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Abstract: BACKGROUND: Imexon is an aziridine-containing small pro-oxidant molecule with promising antitumor activity in myeloma, lymphoma and lung and pancreatic cancer. Imexon is already in clinical trials in patients with advanced solid tumors. The present study examined the effects of imexon on H9 and Raji lymphoma cell lines in vitro when given in combination with ionizing radiation. MATERIALS AND METHODS: H9 and Raji lymphoma cells were grown in culture and exposed to imexon, radiation, or both. Cells were assessed for cell viability, glutathione content, induction of apoptosis, cell cycle distribution and also subject to Western blot analysis. RESULTS: Imexon inhibited cell proliferation in a dose-dependent manner. Imexon, given for 48 h prior to irradiation at a clinically achievable dose of 40 muM, potently enhanced the cell radiosensitivity. Imexon enhanced radiation-induced apoptosis and accumulated cells in G2/M phase of the cell cycle. Imexon induced caspase-3 activation and PARP cleavage. Alterations in glutathione levels were not observed at 40 microM of imexon. CONCLUSION: In conclusion, imexon efficiently augmented lymphoma cell radiosensitivity independently of glutathione and the underlying mechanisms include induction of apoptosis and cell cycle redistribution.

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Imexon Augments Sensitivity of Human Lymphoma Cells to Ionizing Radiation: *In Vitro* Experimental Study

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Abstract. *Background: Imexon is an aziridine-containing small pro-oxidant molecule with promising antitumor activity in myeloma, lymphoma and lung and pancreatic cancer. Imexon is already in clinical trials in patients with advanced solid tumors. The present study examined the effects of imexon on H9 and Raji lymphoma cell lines in vitro when given in combination with ionizing radiation. Materials and Methods: H9 and Raji lymphoma cells were grown in culture and exposed to imexon, radiation, or both. Cells were assessed for cell viability, glutathione content, induction of apoptosis, cell cycle distribution and also subject to Western blot analysis. Results: Imexon inhibited cell proliferation in a dose-dependent manner. Imexon, given for 48 h prior to irradiation at a clinically achievable dose of 40 μM, potently enhanced the cell radiosensitivity. Imexon enhanced radiation-induced apoptosis and accumulated cells in G2/M phase of the cell cycle. Imexon induced caspase-3 activation and PARP cleavage. Alterations in glutathione levels were not observed at 40 μM of imexon. Conclusion: In conclusion, imexon efficiently augmented lymphoma cell radiosensitivity independently of glutathione and the underlying mechanisms include induction of apoptosis and cell cycle redistribution.*

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Key Words: Imexon antioxidant, antiproliferative activity, lymphoma cells, ionizing radiation, radiosensitization.

Imexon (4-imino-1,3-diazabicyclo-[3.1.0]hexan-one) is a 2-cyanoaziridine-containing iminopyrrolidone (AmpliMed Co., AZ, USA) with *in vitro* and *in vivo* activity against a variety of hematological and solid malignancies (1-5). In severe combined immune-deficient (SCID) mice, imexon reduced development of human lymphoma (6). Imexon was also active against a murine tumor model bearing either P-388 or L-1210 leukemia (5). The mechanism of anticancer activity of imexon is not completely understood. In the 1970s and 1980s, imexon was under clinical development as a nonspecific immunomodulatory agent for the treatment of cancer and acquired immunodeficiency syndrome (AIDS) (7-9). In a study using fresh human tumors in an *in vitro* tumor colony assay, imexon showed selective toxicity against human myeloma with an inhibitory concentration (IC_{50}) of 0.2 μg/ml (10). Imexon was subsequently investigated in preclinical myeloma models for its capacity to modulate oxidative stress. In RPMI 8226 myeloma cells, Dvorakova *et al.* (11-13) showed that imexon at concentrations between 90 μM and 500 μM induced apoptosis mediated by formation of reactive oxygen species (ROS) and reduced intracellular glutathione (GSH). Two recent studies using the same RPMI 8226 myeloma model demonstrated differential, concentration-dependent effects of imexon (14, 15). In these studies, low drug concentrations (below 180 μM) induced apoptosis, necrosis or G_2/M cell cycle arrest independently of ROS and GSH levels. Of clinical relevance, imexon is currently being explored for its potency to enhance the effect of classic anticancer agents. Importantly, imexon was active in dexamethasone- and chemotherapy-sensitive and -resistant myeloma cell lines (14). In this study, significant cytotoxicity was observed after 48-hour imexon incubation (80-160 μM) in all cell lines in a time and dose-dependent

manner. The mechanism of imexon cytotoxicity was induction of apoptosis mediated by cleaved caspase 3. Moreover, some but not all cell lines demonstrated caspase 8- and/or bcl2:bax-dependent apoptosis. Another study evaluated the cytotoxic effects of imexon in A375 human melanoma and RPMI 8226 myeloma cells when combined with a broad panel of chemotherapeutic drugs (16). Imexon was synergistic when combined with DNA-binding agents (cisplatin, dacarbazine, melphalan) and pyrimidine-based antimetabolites (cytarabine, fluorouracil, gemcitabine) in both cell lines. The authors speculated that the synergy seen for imexon and alkylating agents may relate to the thiol-dependent sulfhydryl-lowering effect of imexon (17), which would render cells more sensitive to electrophilic species from the alkylators (18). Imexon has been shown to reduce stores of reduced sulfhydryls in RPMI 8226 myeloma cells exposed to high drug concentrations (11). Interestingly, imexon exhibited an antagonistic effect when combined with topoisomerase I and II inhibitors. The authors attributed this antagonistic effect to potentially increased ROS levels, which are known to antagonize the cytotoxic effects of topoisomerase poisons (19). Together, these studies evoked our interest to test the efficacy of imexon to radiosensitize tumor cells. Because radiation, in addition to chemotherapy, is a mainstay in the treatment of human lymphoma we investigated the combined effects of imexon and radiation in two human lymphoma cell lines.

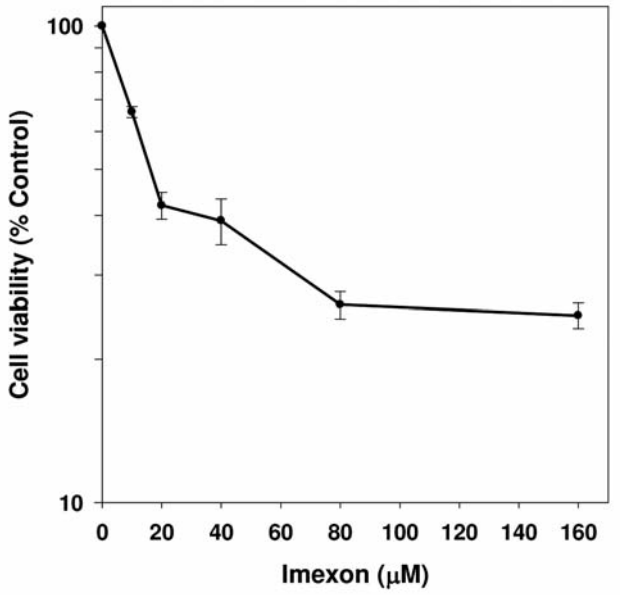

Figure 1. *In vitro proliferation assay in H9 cells. Cells were plated in 96-well plates, then 24 hours later treated with increasing doses of imexon (10-160 μM) and incubated for 48 hours. Cells were then stained with MTT and lysed, and the absorbency was read in a plate reader using a 540-nm filter. Viable cells were calculated as percentage of the control. Data shown are mean±SE from three independent experiments.*

Materials and Methods

Reagents. Imexon used in this study was from Dr. Evan M. Hersh (AmpliMed Co. AZ, USA). Cell culture media and penicillin-streptomycin were from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines. Two human lymphoma cell lines, H9 (T-cell lymphoma) and Raji (B-cell non-Hodgkin's lymphoma), were used for the study. The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and with 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2.

Proliferation assay. Cells were plated in 96-well plates and treated the next day with different doses of imexon (10-160 μM). After 48 hours' incubation, cells were stained with MTT, dissolved in DMSO, and the absorbance was read at 540 nm using a 96-well plate reader.

Cell survival assay (limited dilution assay). The survival assay was performed as described previously (20) with some modifications. Briefly, cells were plated in 96-well plates, incubated with imexon for 48 hours and exposed to 1, 2.5, or 5 Gy of radiation (γ-rays, using a ^{137}Cs source, dose rate 3.7 Gy/min). To test the influence of treatment sequence, additional assays were performed where imexon was added immediately after radiation, or 6 hours after radiation, and maintained for 48 hours. Immediately after radiation, the cells were harvested, plated into 96-well plates and maintained for 14 days in normal growth medium to determine cell growth. Survival was calculated by an adaptation of the estimation of the 50% endpoint between live and dead cells (21). After 14 days, lymphoma cells were stained with MTT and wells with live cells were scored and the quantitative data were fitted by a logistic regression to determine the number of cells needed to achieve growth in 50% of the wells at the respective dose level (D_{50}). The survival fraction at a given dose was calculated as a ratio of D_{50} of irradiated cells and D_{50} of unirradiated, control cells. Survival curves were constructed from at least three independent experiments by fitting the average survival levels.

Glutathione (GSH) assay. Intracellular reduced GSH was assayed in H9 cells by ortho-phthalaldehyde (OPA) staining procedure and the intensity was measured using a fluorometer, as described previously (22) with some modifications. Briefly, cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in ice-cold lysis buffer (5% TCA, 1 mM EDTA, and 0.1 M HCl at 1:1:1, v/v/v ratio). The insoluble materials were removed by centrifugation. GSH measurement was carried out by mixing 0.2 ml lysates with 3.6 ml 0.1 M phosphate/5 mM EDTA buffer (pH 8.0) and 0.2 ml OPA stock (1 mg ml^{-1} ethanol). Fluorescence was measured at 420 nm (emission) with 350 nm excitation wavelength. A standard curve was constructed using known amount of GSH and used for obtaining the GSH levels in the lysates.

Apoptosis and cell cycle distribution. H9 Cells were subjected to TUNEL [terminal deoxynucleotidyl-transferase (TdT) dUTP nick-end labeling] assay and stained with propidium iodide after 48-hour imexon (40 μM) incubation and/or radiation (5 Gy). Apo-direct kit

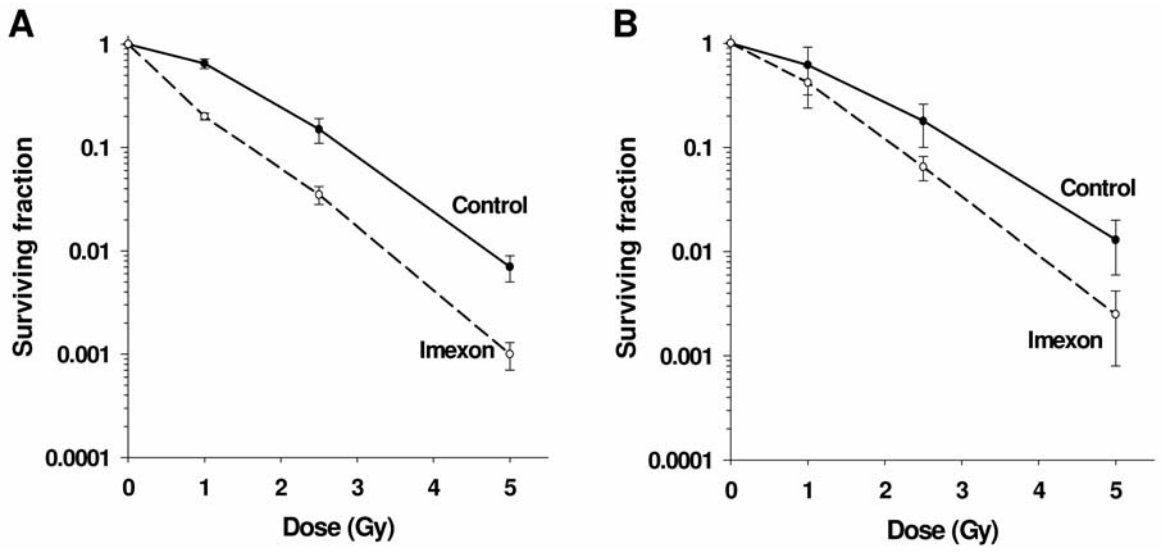

Figure 2. *In vitro cell survival assay (limited dilution assay) of H9 (A) and Raji (B) cells. Cells were pre-incubated with 40 μM imexon for 48 hours and then irradiated. Immediately after irradiation, cells were trypsinized, plated in 96-well plates and allowed to grow for 14 days. Wells that were positive for lymphoma cells were scored and the quantitative data were fitted by a logistic regression to determine the number of cells needed to achieve growth in 50% of the wells at the respective dose level (D_{50}). The survival fraction at a given dose was calculated as a ratio of D_{50} of irradiated cells and D_{50} of unirradiated, control cells. Survival curves were constructed with normalized values for the cytotoxicity induced by imexon alone. Values shown are the means±SE for three independent experiments.*

(BDPharmingen, San Diego, CA, USA) was used following the manufacturer's protocol to quantify the apoptotic cells. The significance of data on apoptosis was analyzed by Student's *t*-test and *p*-value equal to or below 0.05 was considered as significant.

Western blot analysis. Cells were treated with imexon and/or radiation (5 Gy). Cell lysates were obtained at various time points and subjected to Western blot analysis as described (23). Primary antibodies were bought from Abcam Inc. (Cambridge, MA, USA) and secondary antibodies were bought from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The immunoreaction was visualized by ECL plus detection system (Amersham, Arlington Heights, IL, USA) and analyzed using ImageQuant software and Typhoon scanning system (Molecular Dynamics Inc. CA, USA).

Results

Enhancement of radiosensitivity by imexon. Firstly, the antiproliferative effect of imexon, given as a single agent, in H9 cells was determined. Imexon reduced cell proliferation and the effect was dose dependent (Figure 1). A 40% reduction in cell proliferation was observed at the dose of 40 μM of imexon. For further investigation we used 10 and 40 μM of imexon. To determine whether imexon increases the sensitivity of tumor cells to radiation, *in vitro* cell survival was assayed. H9 and Raji cells were exposed to 10 or 40 μM imexon for 48 h and then exposed to increasing doses of radiation (1, 2.5 or 5 Gy). Whereas 10 μM imexon had no effect on cells radiosensitivity (data not shown), 40 μM imexon potently enhanced the radiosensitivity of both the cell lines tested. Figure 2 shows the radiation dose-response curves for H9 and Raji cells. The curves representing the effect of imexon plus radiation were normalized against drug effect. Radiation itself caused a dose-dependent reduction in cell survival. Pretreatment with imexon efficiently enhanced radiosensitivity that was apparent by the shifting of survival curves to left. Radiosensitivity enhancement factors (EFs) were calculated by dividing the radiation dose of the control curve by that of the corresponding imexon plus radiation curves at 0.1 survival fraction. In both cell lines, radiation enhancement was significant (*p*-values Student's *t*-test <0.05): the EF was 2.2 in H9 cells and 1.6 in Raji cells. To investigate whether radiosensitization by imexon was possible if imexon was applied after radiation, additional cell survival assays were performed, where imexon was added immediately after radiation or after a 6-hour time span and incubation was continued for 48 hours. Imexon did not induce radiation enhancement in either experimental set-up (data not shown).

Induction of apoptosis. Apoptosis is an important mechanism of cell death in hematological malignancies. To investigate whether the combined effect of imexon and radiation was due to increased apoptosis, H9 cells were subjected to TUNEL assay. Since 40 μM of imexon was effective in enhancing cell radiosensitivity, induction of apoptosis was tested after exposing the cells to 5 Gy of radiation, 40 μM of imexon for 48 hours, or both where cells were first exposed to imexon for 48 hours prior to irradiation. Cells

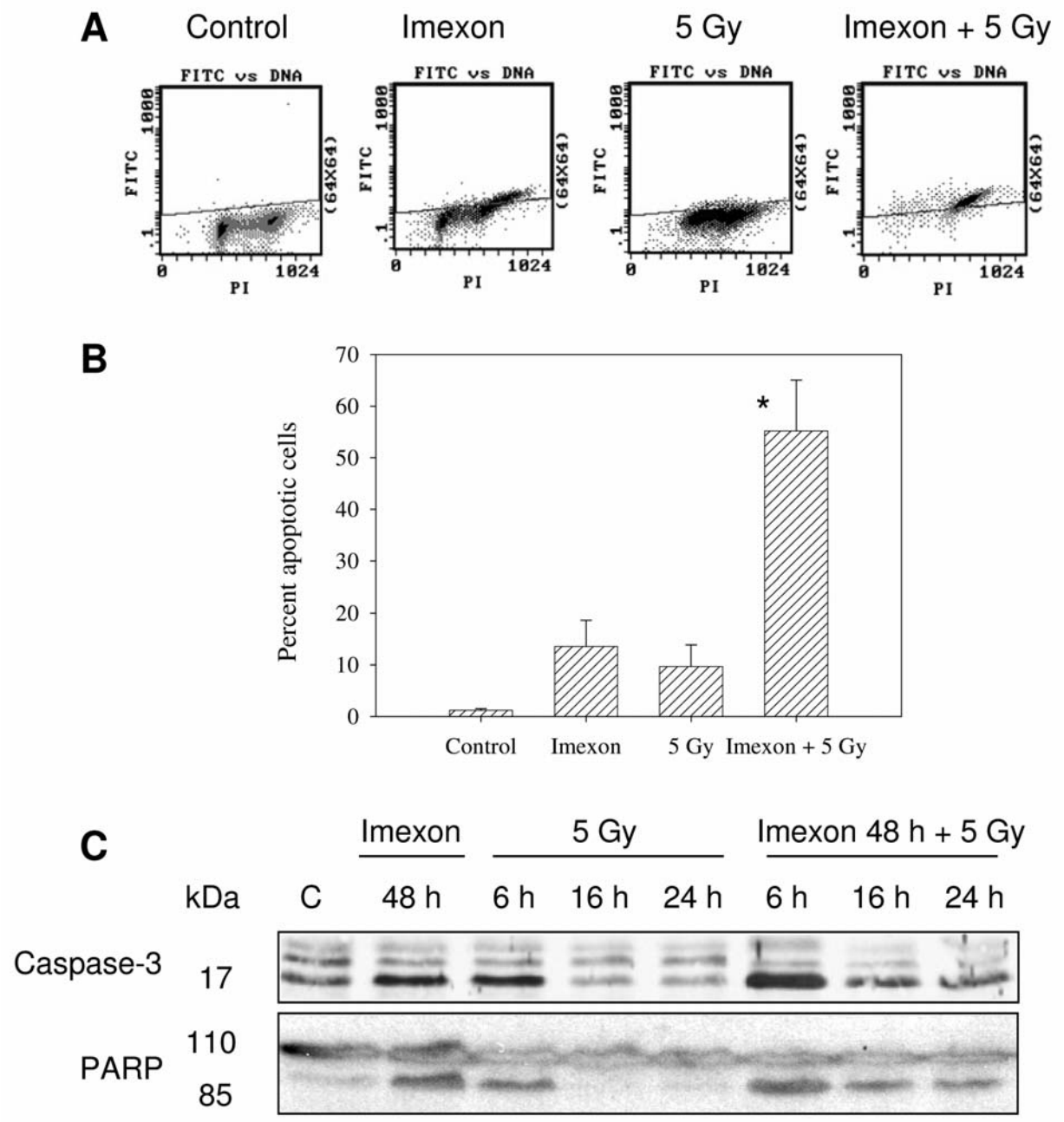

Figure 3. *Induction of apoptosis by imexon and radiation in H9 cells. Cells were pre-incubated with 40 μM imexon for 48 hours before exposing them to a single dose irradiation (5 Gy). Cells were subjected to TUNEL assay 24 hours after irradiation. A, Flow cytometric analysis of apoptotic cells. Data are representative of three independent experiments. B, Quantitative data on apoptosis induced by imexon and radiation from three independent experiments (mean±SE). * Significant supra-additive effect of combined treatment (Student's t-test, p≤0.05). C, Western blot analysis for the expression levels of cleaved caspase 3 and PARP. Cells were lysed at specific time points shown in the figure after treatments with imexon, radiation or both. Whole cell lysates were subjected to Western blot analysis. Representative blots of three independent experiments are shown.*

were assayed 24 hours after irradiation. Imexon and 5 Gy when given as single agents induced apoptosis by 13.6±5% and 9.6±4.2%, respectively. When these two agents were combined, the induction of apoptosis was supra-additive, 55.2±9.8% (Figure 3A, B).

Western blot analysis. To determine alterations in cellular proteins that are involved in apoptosis, whole cell lysates of H9 cells were prepared after imexon (40 μM, 48 hours) and radiation treatment and were subjected to Western blot analysis. Imexon or 5 Gy as single agent induced cleavage of

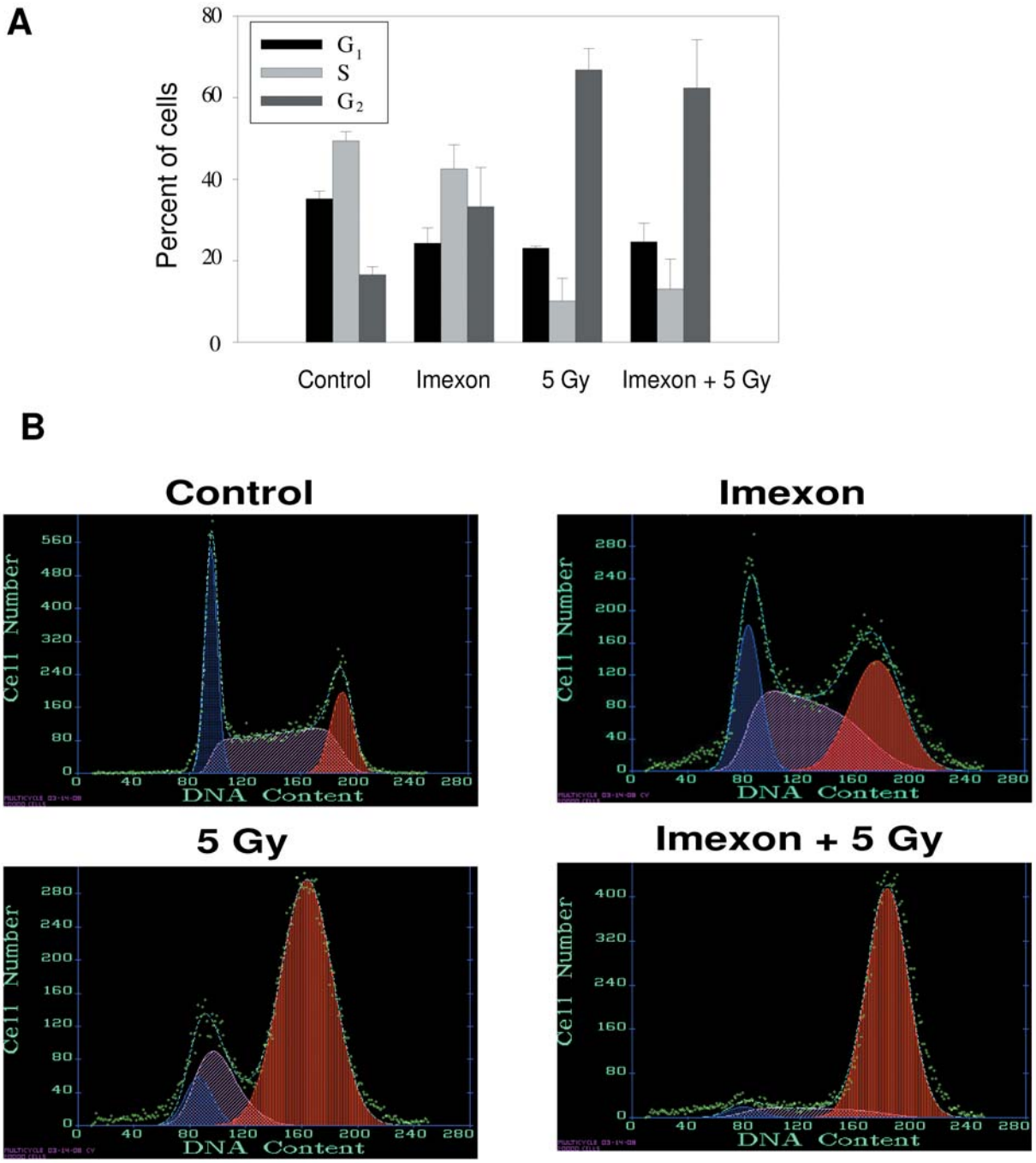

Figure 4. *Cell cycle redistribution in H9 cells. A, Cells were exposed to imexon, radiation (5 Gy), or both, then fixed and stained with propidium iodide (DNA-stain)/RNase solution and analyzed by flow cytometry. Data shown are means±SE of three independent experiments. B, Representative cell cycle analysis after imexon, radiation, or both. Imexon caused accumulation of H9 cells in the radiosensitive G_2 phase of the cell cycle while reducing the number of cells in the G_1 and S phases.*

caspase-3, which was significantly enhanced when both treatments were combined (Figure 3C). To investigate if apoptosis induction was mediated *via* mitochondrial (caspase-9) or death receptor (caspase-8) pathway, we performed Western blotting against cleaved caspase-9 and caspase-8. A moderate increase in cleaved caspase-9 was observed after imexon (48 hours) treatment. Exposure of cells to 5 Gy and analysis of cell lysates 6 hours after irradiation showed a slight

increase in cleaved caspase-9 protein; however, combined treatment with imexon and irradiation failed to induce caspase-9 cleavage at the time points tested. H9 cells did not show any measurable amount of caspase-8 (data not shown).

Effect on the cell cycle. Previous reports indicated that imexon affects the cell cycle distribution of myeloma cell lines at low doses. Because the cell cycle distribution has an effect on radiation sensitivity, we performed cell cycle analysis after 48 hours of incubation with imexon±radiation. As shown in Figure 4, imexon caused H9 cells to accumulate in the radiosensitive G_2 phase of the cell cycle while reducing the number of cells in G_1 and S phases.

Effect on cellular redox status. Previous studies link the pro-apoptotic effect of imexon in myeloma cell lines to modulation of the cellular redox status; however, high concentrations of imexon were used in these studies (>90 μM) (11-13). To investigate whether radiosensitization by imexon in H9 cells was mediated by alterations in cellular redox status, we assessed reduced GSH levels after exposing the cells to imexon (40 μM) for 48 hours, after radiation, or after both. Imexon slightly reduced the GSH level in H9 cells but this effect was not significant (data not shown).

Discussion

In this study, we explored the efficacy of imexon at enhancing the sensitivity of H9 cells and Raji cells to radiation. We found that both lymphoma cell lines were sensitive to imexon in a dose range that is comparable to myeloma cell lines investigated in other reports (IC_{50} below 50 μM) (14). Our study showed that enhancement of radiosensitivity in H9 cells by imexon was dependent on the sequence of administration. The greatest extent of radiosensitization was achieved when imexon was given for 48 hours before the radiation. It is interesting to note that imexon was not effective in enhancing radioresponse when given after radiation. Pretreatment with imexon accumulated cells in the G_2 phase, a radiosensitive phase of the cell cycle (24). Thus, imexon may have increased the susceptibility of cells to radiation-induced cell death. The cellular redox system has been demonstrated to be involved in cellular resistance of hematological malignancies (25-28). One line of evidence indicates that imexon-induced apoptosis correlates with formation of reactive oxygen species, depletion of thiols and mitochondrial alterations at high concentrations between 90 and 500 μM in several studies (11, 12, 14, 15). GSH is a non-protein cellular thiol that is a critical component to cellular oxidative stress (29, 30) and has been found to sensitize tumor cells to oxidative cytolysis (31-33). Evens *et al*. (14) suggested that imexon-induced apoptosis is independent of ROS production and thiol depletion at low dose levels (80-160 μM). In their study using various dexamethasone and chemotherapy-sensitive and -resistant myeloma cell lines, significant ROS production and thiol depletion occurred only at imexon concentrations >300 μM after 48 hours incubation and they did not correlate with apoptosis. Consistent with this study, our data showed no significant thiol depletion after 40 μM of imexon treatment in the lymphoma cells. It is important to note that in the aforementioned study (14), imexon exhibited its effect on refractory lymphoma cells.

Apoptosis is induced either by intrinsic or extrinsic pathway (34). Dvorakova *et al*. (3) reported that imexon-induced redox regulation activates the intrinsic or mitochondrial pathway of apoptosis involving cytochrome *c* release and activation of caspase-9 and -3. Caspase-9 has been proposed as the major caspase in the mitochondrial pathway of apoptosis that is death receptor independent. Evens *et al*. (14) described increasing proapoptotic bax expression and alteration of the bcl-2/bax ratio after treatment with low doses of imexon. Furthermore, they showed that in some myeloma cell lines, imexon-induced apoptosis was caspase-8 dependent. Our data showed that imexon activated caspase-9 and -3, while activation of caspase-8 was not observed, suggesting that imexon predominantly initiated the intrinsic, mitochondrial pathway of caspases in H9 cells.

Therapeutic implications for the treatment of lymphoma are that imexon could be effective in malignant lymphoma that lacks receptor for the activation of apoptosis (3). In a recent clinical phase 1 dose escalation trial in patients with advanced malignancies, grade 3 abdominal pain and fatigue, and grade 4 neutropenia were observed as dose-limiting toxicities of imexon (35); interestingly, a decrease in plasma thiol levels did correlate with imexon exposure. Our study presents evidence that 40 μM imexon, which is non-toxic and clinically achievable, can be combined with radiotherapy to improve the treatment outcome. In conclusion, our *in vitro* data presented here show that imexon potently enhanced the radiosensitivity of H9 and Raji cells, and the underlying mechanisms may include apoptosis and cell cycle redistribution. These data suggest that imexon has the potential to increase the radioresponse of malignant lymphoma and warrants further investigation using *in vivo* tumor models.

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